Studies in the Fat Metabolism of the Timothy Grass Bacillus.

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(From the Biochemical Laboratory, Cambridge.)

Studies in fat metabolism have hitherto been chiefly carried out on highly specialised vertebrate tissue. By making investigations on a unicellular organism, more susceptible of laboratory control, two objects were in view: (1) to trace the stages by which the long straight chains of the fatty acid molecules are built up from the constituents of the nutritive medium; (2) to follow the circumstances and course of their subsequent breakdown. The Timothy grass bacillus was the organism selected, on account of (1) its high content of fat, and (2) its relationship to the tubercle bacillus, any facts substantiated by study of the one probably helping to throw light on the chemical habits of the other.

METHODS OF CULTURE AND ANALYSIS.

The medium employed contained inorganic salts in the following proportions:—

Potassium phosphate (K_2HPO_4)	0·1 grm. per 100 c.c.
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	0·07 „ „
Ammonium phosphate (Am_2HPO_4)	0·4 „ „
Calcium carbonate	excess.

together with traces (about 0·006 grm. per 100 cubic centimetres) of sodium chloride, introduced when the organism was sown. The calcium carbonate was used to maintain the P_H of the medium at a constant value, namely, about 8·0. The ammonium salt formed the only source of nitrogen. The source of carbon varied with different experiments and will be discussed later; nothing more complex than glucose was employed.

Various types of culture vessel were tried, but the only one with which we were able to attain any degree of success was the Roux bottle. Attempts were made to use flasks or large bottles, with rubber stoppers fitted with delivery tubes through which samples of the medium could be withdrawn at intervals. Numerous attempts all proved completely unsuccessful for the following reasons:—(1) The organism grows mainly in a scum on the surface of the medium. If a deep vessel is employed this scum is far removed from the layer of chalk on the bottom, which consequently fails in its function. For this or other reasons connected with aeration, the growth of the organism slows down and comes to a standstill long before the organic constituent of the medium is used up. (2) The withdrawal of samples by means of delivery tubes plugged with cotton-wool proved completely unsuccessful. When the medium was shaken in order to distribute the growth evenly before taking a sample, the scum formed clots or flakes which tended to float, making it impossible to blow out a fair sample of medium through the delivery tube.

The method finally adopted was identical with that first employed, viz., culture in Roux bottles. The bottles, each containing about 5 grm. of calcium carbonate, were plugged in the usual way and baked for an hour at 130°–140°. Exactly 150 c.c. of medium were then introduced into each bottle, and the set of bottles was steam-sterilized on each of three successive days.

The culture was first sown on glycerol potato slopes and incubated for four to seven days. A number of test-tubes (6 inch by 1 inch) containing a small quantity of clean sand, together with about 5 c.c. of saline, were plugged and autoclaved. Into one of these the growth from the potato slopes was removed by a platinum loop. The growth from one potato slope was sufficient for two or three Roux bottles. When sufficient growth had been removed to sow the required number of bottles, it was emulsified.

The emulsification of this organism presents difficulties. The apparatus shown in fig. 1 was employed. A is a solid glass club, the handle of which passes through tubes B and C. These are arranged to form a mercury seal. The rubber stopper D is fitted into a test-tube of the same size as those containing the sterile sand and saline, and the whole is wrapped in paper and sterilized in an oven at 120°–130° for an hour. Stopper D is then removed from the empty tube. At the same moment the cotton-wool plug is removed from the tube containing the organism, sand and saline, and this tube

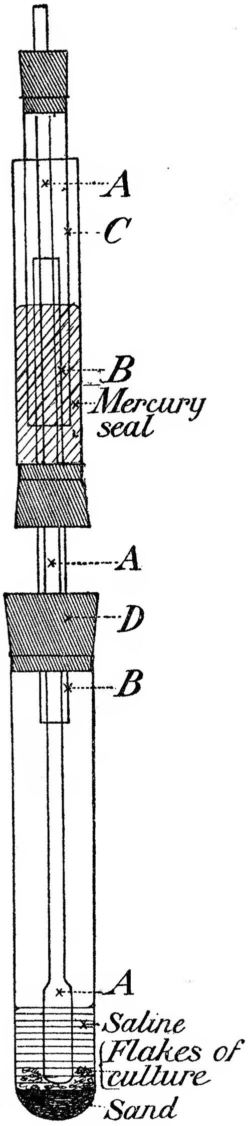


Fig. 1.

immediately replaces the empty one, and is closed by stopper D. Mercury (kept under 5 per cent. formalin) is then introduced by means of a sterile curved pipette to form a seal and prevent contamination of the tube whilst allowing sufficient play to the glass rod. The pieces of growth can be ground in the sand and a fairly even emulsion formed. When this is complete, the club and seal are withdrawn with the stopper D and the cotton-wool plug is replaced in the test-tube, after about 25 c.c. of sterile saline have been introduced and mixed with the emulsion. To avoid sowing a contaminated culture, the diluted organism was then incubated for 24 hours and sown on to slopes of tryptic agar, which medium favours the rapid growth of contaminating organisms. After 24 hours' incubation, the growth on these slopes was examined by the Ziel-Neelsen method of staining. If satisfactory, the original emulsion was sown by means of sterile pipettes, 1 c.c. to each Roux bottle. We have employed this method for a large number of experiments and have only once suffered from contamination, which was detected on the slopes before the bottles were sown.

The bottles thus sown were incubated at 37°, and sample bottles were withdrawn at intervals in order to analyse both the medium and the organism grown upon it. It will be seen that the success of the experiment depends on introducing into each bottle an equal amount of the emulsified culture. The analysis of duplicate Roux bottles withdrawn simultaneously shows agreement within the limits of accuracy of the analytical methods used, as will be shown later.

Two methods are most frequently used to estimate the amount of growth of any organism: (1) plating out and counting colonies; (2) separation of the growth by centrifuging, washing, drying, and weighing direct. The former was impossible in our experiments owing to the agglutinated character of the growth; the latter was equally unsuitable, since the growth was always mixed with the powdered calcium carbonate employed to control the reaction of the medium. In our experiments, therefore, we regarded the protein nitrogen synthesized as a measure of the growth of the organism. This protein nitrogen was precipitated by colloidal iron and estimated by Kjeldahl's method. The other characteristic of the organism to be examined was its fat content. The lipoid material of the bacillus may be conveniently divided into two: fraction A is that extracted from the dried organism by purified ether; this fraction contains no phosphorus. From the residue after extraction of A is obtained fraction B, by treatment with boiling alcohol and a subsequent second extraction with ether; B contains phosphorus. For convenience fraction A has been termed the "fat" fraction, B the "phosphatide" fraction, and the sum of the two "total lipoid."

Estimation of Synthesised Protein.

The contents of one Roux bottle were washed quantitatively into a beaker and made slightly acid with acetic acid; 20 c.c. of "dialysed iron" were added and the whole heated on a water-bath, filtered whilst hot, and washed with hot water till free from phosphate. The precipitate and paper were then transferred to a Kjeldahl incinerating flask and the nitrogen estimated in the usual manner. The results are calculated to represent grm. of nitrogen in 100 c.c. medium, *i.e.*, two-thirds of one Roux bottle.

Estimation of Lipoids of Bacillus.

(1) "*Fat.*"—The contents of one Roux bottle (150 c.c.) were transferred quantitatively to a 250-c.c. measuring flask, and made up to volume. They were then filtered through a dry pleated filter into a dry flask, and the filtrate was set aside for estimation of the constituents of the medium. The precipitate, consisting of the insoluble constituents of the medium together with the agglutinated bacteria, was transferred on the filter paper to a dish and dried in an atmosphere of nitrogen at about 97°. The final stages of the drying were carried out *in vacuo*. The filter paper and dried contents were then transferred to a Soxhlet extractor and extracted with ether (purified from alcohol and aldehydic substances). The extraction flasks employed, of about 150 c.c. capacity and about 35 grm. in weight, were fitted to the extractor with ground glass joints, in order to get good quantitative results. After about 8 hours' extraction, the ether was distilled off and the flask dried in nitrogen and later *in vacuo* at 97°. The extract thus obtained represents the "fat" fraction described above.

(2) "*Phosphatide.*"—The Soxhlet thimble and contents were then transferred to a wide-mouthed extraction flask fitted with a condenser. To this about 30 c.c. of alcohol (previously distilled from caustic soda) were added; the alcohol was boiled on a water-bath for 20 minutes and then distilled off and the flask and contents dried in an atmosphere of nitrogen. The small amount of material which had dissolved out of the thimble was then washed back with ether and the thimble and contents were returned to the Soxhlet extractor; a second extraction was then made with ether and the flask dried and weighed as before. This extract is the "phosphatide" fraction referred to above.

No detailed chemical examination of these fractions has been made, but it has been shown that the "fat" fraction contains no phosphorus, whilst the "phosphatide" fraction contains a small but fairly constant quantity (estimated by Neuman's method). Both fractions were tested for nitrogen by Kjeldahl's method: traces (below 1 per cent.) were found in both fractions.

Phosphorus Estimation.

Weight of "phosphatide" fraction taken; in grm.	Mgrm. of phosphorus found.	Percentage of phosphorus.
0·0280	0·346	1·23
0·0284	0·382	1·34
0·0240	0·270	1·12
0·0241	0·270	1·12

Nitrogen Estimation.

Weight of lipoid taken; in grm.	Mgrm. of nitrogen found.	Percentage of nitrogen.
0·1126 ("fat")	0·989	0·78
0·1260 ("phosphatide")	0·823	0·73

Probably both fractions are complex mixtures of lipoid bodies similar to those obtained from the tubercle bacillus [Goris, Goris and Liot, 1920 (1)]. The two are treated separately in this research on account of the absence of phosphorus in fraction A and its presence in fraction B.

Analysis of the Medium.

As described above, the contents of one Roux bottle (150 c.c. of original medium) were made up to 250 c.c., from which the agglutinated bacteria and solid mineral matter (chalk, etc.), were then removed by filtration. The filtrate was then analysed for glucose or other carbon compound present. Glucose estimations were carried out on 5 c.c. of the diluted medium by the method of Wood-Ost [Wood and Berry, 1903, (4)]. Ammonia was estimated in the first and last samples by the method of Van Slyke [1911, (3)], in order to ensure that growth of the bacteria had not ceased through lack of nitrogen.

Experiment 1.—*Growth of the Bacillus in respect of Total Nitrogen and Lipoids on the usual Inorganic Medium, containing as well* 1 *per cent. Glucose and* 1 *per cent. Acetic Acid, added as Sodium Acetate.*

Twenty-five Roux bottles, prepared and inoculated as described above, were incubated at 37° and bottles were withdrawn at intervals in sets of two, one for the estimation of total nitrogen, and the other for the estimation of lipoids and of the organic constituents of the medium. The disappearance of acetic acid was not followed stage by stage in this first experiment, but it was ascertained that by the time the glucose had been utilised the acetic acid had also disappeared.

The reaction of the medium was kept constant by chalk, which was spread over the bottom of the Roux bottles; the P_H at the beginning, end, and all intermediate stages remained 8·0. This was tested by phenol red, and kindly checked for us by the hydrogen electrode by Miss Jordan Lloyd.

The figures given in column 1 of the following table represent the actual amounts obtained from single Roux bottles (150 c.c. each), which were of necessity the units employed; column II represents the same amounts calculated for 100 c.c. medium, from which the points on Curve 1 are taken. Where estimations were made on duplicate Roux bottles, the figures are bracketed.

Fat Synthesised.

Day of experiment.	"Fat" fraction. Weight in grm.		"Phosphatide" fraction. Weight in grm.		Total lipoids. Weight in grm.	
	I.	II.	I.	II.	I.	II.
7*	0·0054	0·0036	0·0083	0·0054	0·0137	0·0090
14	0·0198	0·0132	0·0181	0·0120	0·0379	0·0250
21	0·0399 0·0348	0·0288	0·0284 0·0280	0·0188	0·0623 0·0625	0·0420
22	Organic constituents of the medium completely utilised.					
28	0·0179 0·0268	0·0149	0·0240 0·0241	0·0160	0·0419 0·0501	0·0310
36	0·0145	0·0096	0·0230	0·0152	0·0375	0·0124
60	0·0089 0·0095	0·0061	0·0162 0·0152	0·0105	0·0251 0·0247	0·0170

* The results obtained on the seventh day are too small relatively to the experimental error to be important. They merely serve to indicate that growth had begun.

Protein Nitrogen Synthesised.

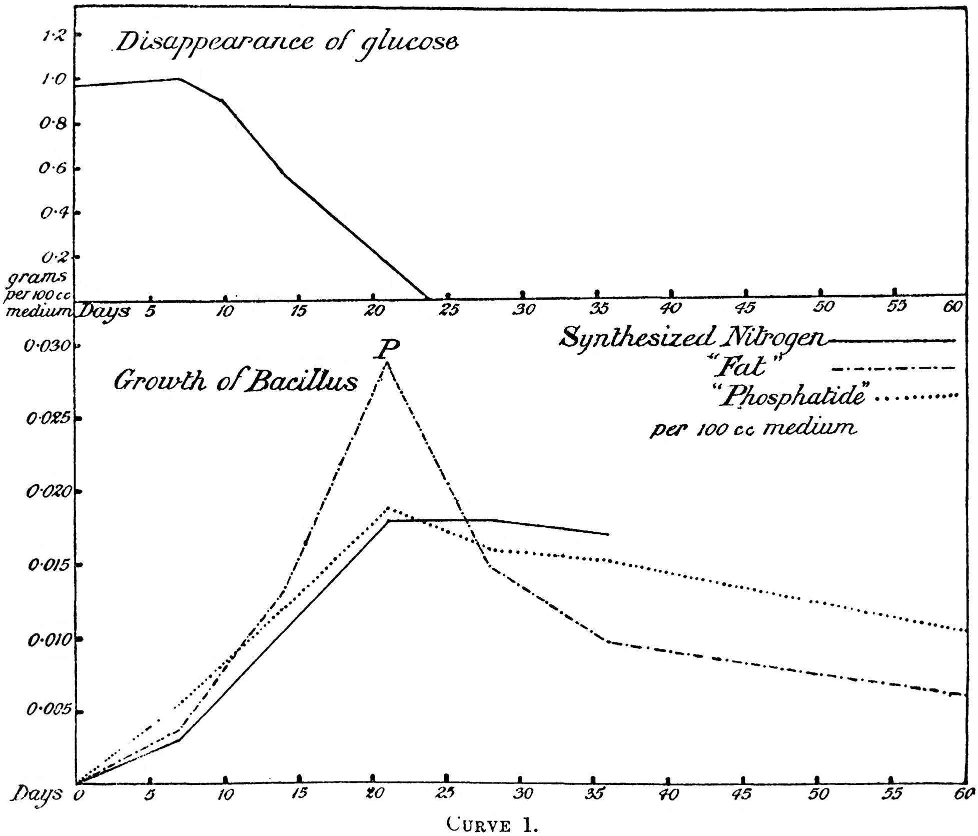
Day of experiment.	Grm. per Roux bottle. I.	Grm. per 100 c.c. medium. II.
7	0·005	0·003
21	0·027 0·027	0·018
28	0·027	0·018
36	0·026	0·017

Glucose Utilised.

Day of experiment.	Glucose present; grm. in 100 c.c. medium.	Glucose utilised; grm. in 100 c.c. medium.
0	0·97	0·00
7	1·00	0·00
10	0·90	0·07
14	0·58	0·39
21	0·14	0·83
28	0·00	0·97

NOTE.—On the 28th day the acetic acid also had completely disappeared.

It is apparent from Curve 1 that the growth of the bacillus, as measured by protein nitrogen and lipoids synthesised, reaches a maximum at the point when glucose and acetate disappear from the medium. From this point onwards, the bacillus utilises its own lipoid material, which rapidly decreases. It is particularly noteworthy that the "fat" fraction decreases at first more



CURVE 1.

rapidly than the "phosphatide" fraction, until an equilibrium is established in which the "phosphatide" fraction is maintained at the higher value. This suggests that the "fat" fraction represents a form of stored food material which is drawn upon when external sources of carbonaceous food material fail, whilst the "phosphatide" fraction, which decreases less rapidly, is probably partially composed of some unit essential to the chemical structure of the cell. The duplicate determinations of the "fat" fraction on the twenty-eighth day show a wide variation. This is in the period of rapid utilisation of stored fat. A slight retardation of the point at which maximum growth was attained and utilisation of fat began would result in a large observable difference at this point.

The results of starvation thus show that the fat metabolism of the higher animals has its prototype in this micro-organism.

It also appears from the curve that the protein nitrogen decreases very slightly during the first fourteen days of starvation, the drop between the twenty-eighth and thirty-sixth day being actually within the experimental error. The bacillus thus seems to preserve its protein material intact. It must be remembered, however, that throughout the experiment ammonia was present in the medium; the bacillus may therefore have broken down cell protein as well as lipoid material, and have reconstituted the former from the ammonia of the medium and the fatty acid chains of the lipoids. Unfortunately no material was available for a final protein nitrogen estimation on the sixtieth day.

It will be noticed that there is a small but definite growth of bacillus on the seventh day of the experiment, while the glucose is apparently untouched. We have confirmed this observation repeatedly in experiments where glucose forms the only organic food material. It may have its explanation in the small amount of food material carried over from the potato slopes, or it may be that in the initial breakdown of the sugar molecule a small quantity of a reducing body accumulates which is erroneously estimated as glucose; such a body has been searched for but not as yet found.

During the course of the foregoing experiment, in 100 c.c. of medium, approximately 1 grm. of glucose and 1 grm. of acetic acid (as acetate) disappeared, together with certain unascertained quantities of mineral material and ammonia. Their place was taken by 0·018 grm. of protein nitrogen, corresponding roughly to 0·1 grm. protein, and 0·041 grm. of mixed lipoid material.

In a similar experiment in which glucose was the sole organic constituent of the medium, 1 grm. of glucose gave rise to 0·0189 grm. of protein nitrogen and 0·028 grm. of lipoid material. In an experiment such as the latter, it was hoped that some intermediate products in the change from glucose to fat might be isolated, and experiments with that end in view were made.

Experiment 2.—*Search for Intermediate Products.*

Five Roux bottles, in all 750 c.c. of medium, containing the usual inorganic salts, with calcium carbonate and 1 per cent. glucose, were inoculated in the usual way and incubated for fourteen days. The total contents of the five bottles were made up to 2,000 c.c., and filtered. The glucose was estimated in the filtrate and was found to be 0·11 per cent. (calculated on the original medium). 0·89 per cent. glucose had thus disappeared from the original 1 per cent. The medium still had its original reaction, P_H 8. Half the filtrate (corresponding to 375 c.c. of the original medium) was steam-distilled, first from neutral solution. The distillate was collected in cold water in fractions and examined for iodoform-producing bodies; a slight iodoform reaction was obtained from the distillate collected during the first five minutes, but none from subsequent fractions. The iodoform could only be detected by the smell; no visible precipitate was obtained.

The residue in the distilling flask was then acidified with phosphoric acid and again steam-distilled, the distillate being collected in standard 0·1N baryta (50 c.c.). At the end of 1½ hours the distillation was stopped and the baryta back-titrated with hydrochloric acid, using phenol phthalein. The difference gave the acidity due to volatile acids and carbonic acid, of which a considerable quantity had come over. The carbonate was then filtered off, washed, transferred to a flask, mixed with 50 c.c. 0·1N HCl, and boiled under a reflux condenser fitting into the neck of the flask. The residual acid was back-titrated with baryta. The results from 375 c.c. of the original medium were as follows :—

Carbonate and volatile acids	36·2 c.c.	0·1N baryta.
Carbonate alone	29·4 „	0·1N „
Volatile acids	6·8 „	0·1N „

Calculated as acetic acid this is equivalent to 0·011 per cent. acetic acid in the original medium. The residue from the steam-distillation of the total medium (750 c.c.) was evaporated on a water-bath to about 150 c.c., filtered, and the filtrate extracted with ether in a continuous extractor for 24 hours. The solid matter was dried and extracted in a Soxhlet apparatus. The ethereal extract from the liquid extraction contained no solid organic acids. The aqueous residue left after the evaporation of the ether was tested for lactic acid by Hopkins' thiophene test; lactic acid appeared to be present in traces, but this could not be established with certainty owing to the rapid darkening of the solution by easily charred matter. No solid material was obtained from the Soxhlet extractor.

These results were confirmed by two other experiments, slight variations only being obtained in the amount of volatile acids distilled over. It seemed therefore that, under the conditions prevailing in these experiments, intermediate products in the breakdown of glucose accumulate only in such traces that attempts at identification are hopeless.

Experiment 3.—*An Attempt to Obtain Fermentation apart from the Growth of the Organism.*

Six Roux bottles were filled with a medium containing the usual inorganic salts and 1 per cent. glucose. From six otherwise exactly similar bottles the ammonium phosphate was omitted. These twelve bottles were sown more thickly than usual with the emulsion of the bacillus. At the end of fourteeen days the sugar in the bottles containing no ammonia was estimated and found completely untouched, whilst that in the control bottles had disappeared in the normal manner. It thus appears that under these conditions fermentation does not occur apart from growth, which in its turn is dependent on a supply of nitrogen.

Direct attempts at isolation of intermediate products having failed, indirect evidence was sought by growing the organism on *possible* intermediate products in the breakdown of the sugar molecule, in order to ascertain: (1) whether the organism was capable of utilising and growing on the intermediate products; (2) if growth took place, how the formation of fat was affected.

The formation of protein nitrogen (precipitated by colloidal iron) was taken as a criterion of growth, the lipoids corresponding to the nitrogen synthesised at each period being separately estimated as previously described. At the point of maximum lipoid formation (*e.g.*, point P, curve 1), the growth of the organism was considered to have attained its maximum, and at that point the lipoid formation was compared with the protein nitrogen synthesised.

Experiment 4.—*Growth on, and Utilisation of, Lactic Acid.*

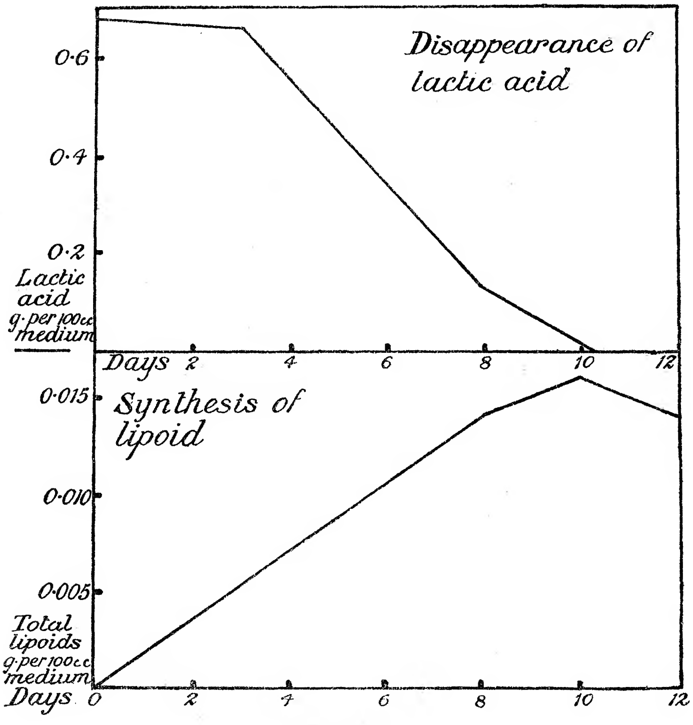
A series of Roux bottles was started in the usual way containing the usual inorganic salts and also 0·68 per cent. lactic acid (dl) added as sodium lactate. Sample bottles were withdrawn at intervals and analyses of the organism carried out as in Experiment 1. Lactic acid was estimated in

25 c.c. samples of the diluted medium by the extraction method of Hopkins and Fletcher [1907, (2)], and weighed as zinc lactate.

The disappearance of lactate from the medium and the synthesis of total lipoids is summarised below and shown graphically on Curve 2.

Day of experiment.	Lactic acid present; grm. in 100 c.c. of medium.	Lactic acid utilised; grm. in 100 c.c. of medium.	Total lipoids formed; grm. in 100 c.c. of medium.
0	0·68	0·00	0·00
3	0·66	0·02	Not estimated.
8	0·13	0·55	0·014
10	0·01	0·67	0·016
12	0·00	Total	0·014

Grm. nitrogen synthesised per 100 c.c. on 10th day, 0·018.



Curve 2.

It will be seen from the above that the organism readily used lactic acid as its sole source of organic food material, and that the course of its growth does not differ materially from that on glucose and acetic acid already established. Similar results were obtained when the lactic acid was added in the form of calcium lactate. The relationship between the total lipoids formed and the total protein nitrogen at the point of maximum growth will be discussed later.

Experiment 5.—*Growth on, and Utilisation of Acetic Acid.*

Roux bottles containing the usual inorganic salts, with 0·5 per cent. acetic acid as sodium acetate, were steam sterilised and sown in the usual way.

Estimation of the Acetic Acid.—The contents of one Roux bottle (150 c.c. of original medium) were made up exactly to 250 c.c., filtered through a dry filter into a dry flask, and the acetic acid then estimated by distillation of 20 c.c. acidified with phosphoric acid. The usual method of estimating acetic acid by steam distillation was found unsatisfactory. The amount of distillate which must be collected before the acetic acid has completely come over is at least 2,000 c.c.; this contains large and variable quantities of carbon dioxide, which must be separately estimated by collecting the distillate in excess standard baryta, neutralising the excess with standard hydrochloric acid, filtering off and separately estimating the barium carbonate. The whole process takes between three and four hours, and gives moreover very variable and unreliable results. Instead, a modified procedure was adopted.

The distillation of the acetic acid was carried out *in vacuo*. The apparatus used was identical with that described by Van Slyke (*loc. cit.*) for the estimation of ammonia, except that in place of the second smaller distilling flask, a thick-walled filtering flask was found more convenient; 10 c.c. of diluted medium (or an amount containing from 0·06 to 0·1 grm. of acetic acid) was placed in a Claissen flask of 300–400 c.c. capacity, fitted with a capillary tube in the first neck, and a tap in the second neck. The liquid was acidified with four or five drops of saturated phosphoric acid. An equal volume of 95 per cent. alcohol (previously distilled from caustic soda) was added. An exact volume (about 25 c.c.) of 0·1N baryta was run from a burette into the distilling flask and the filtering flask, and the capillary tube was adjusted to dip beneath the surface of the liquid. The contents of the Claissen flask were then distilled *in vacuo* (10–15 mm.), and the distillate collected in the baryta in the cooled receiver, any escape of acetic acid being prevented by the baryta in the filtering flask. The distillation was continued until only about 1 c.c. of liquid remained in the Claissen flask. The temperature during the last five minutes should be at least 60°. When the distillation was complete, air was admitted by the capillary and by opening the tap in the second neck of the Claissen flask; 20 c.c. of 50 per cent. alcohol were then poured into the flask and the distillation repeated as before, care being taken that the temperature of the water-bath reached 60° for at least five minutes. The apparatus was then disconnected, the baryta carefully washed into the filtering flask, and the excess titrated with standard hydrochloric acid. A small and variable amount of barium carbonate was always present and was separately estimated. The neutralised liquid was filtered, the solid (barium carbonate) washed twice and then transferred into a flask fitted with a small reflux condenser (preferably ground in); 10 c.c. of 0·1N HCl was then added to the contents of the flask and the whole boiled for five minutes under the reflux condenser. The excess HCl was back-titrated with baryta.

The method was tested as follows:—

10 c.c. of a solution of acetic acid were titrated direct against 0·1N baryta, using phenol phthalein; 10 c.c. of the same solution of acetic acid were distilled into baryta as described above, and the results compared. The following are the figures for the distillation:—

A. C.c. acetic acid taken.	B. C.c. 0·1N $Ba(OH)_2$ taken.	C. C.c. 0·1N HCl used for back titration.	D. C.c. 0·1N $Ba(OH)_2$ neutralised by acetic acid and CO_2 [B–C].	E. C.c. 0·1N $Ba(OH)_2$ neutralised by CO_2 only.	F. C.c. 0·1N $Ba(OH)_2$ neutralised by acetic acid [D–E].	Per cent. of acetic acid in solution.
10	25·8	2·6	23·2	0·7	22·5	1·35
10	25·4	1·6	23·8	1·4	22·4	1·34

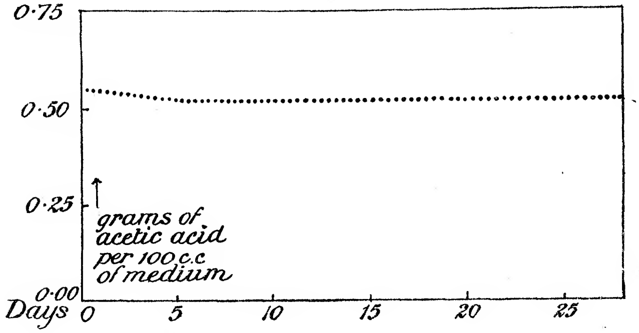
10 c.c. of acetic acid titrated direct = 22·1 c.c. 0·1N $Ba(OH)_2$.

∴ 10 c.c. acetic acid contain 0·133 grm. = 1·33 per cent. compared with 1·35 and 1·34 by the distillation method.

The disappearance of acetic acid from the medium is summarized below, and shown graphically on Curve 3.

Day of experiment.	Acetic acid present; grm. in 100 c.c. of medium.	Acetic acid utilised; grm. in 100 c.c. of medium.
0	0·53	0·00
4	0·49	0·04
28	0·46	0·07

The growth of the organism was too small to admit of accurate estimation. From this it appears that, in the conditions holding in this experiment, the organism suffers from almost complete inability to use acetic acid as a source of organic food material. The slight disappearance of acetic acid is probably due to a small amount of carbohydrate carried over from the potato slopes (see below).



CURVE 3.

Since it has been shown in earlier experiments that no acetic acid accumulates during the growth of the organism on glucose, and since acetic acid has been shown by many workers to be a frequent intermediate product in the breakdown of glucose by bacteria, it seemed worth while to ascertain whether the presence of other substances might not confer on the Timothy grass bacillus the power of utilising this compound.

Experiment 5.—*Utilisation of Acetic Acid in the presence of Lactic Acid or of Glucose.*

Sixteen Roux bottles, containing the usual inorganic medium with 1 per cent. lactic acid (added in the form of calcium lactate) and 0·5 per cent. acetic acid (added as sodium acetate), were treated as described in Experiment 1. Acetic acid was estimated on 20 c.c. of the diluted medium. It was found that a correction had to be introduced owing to a small constant amount of lactic acid distilling over with the acetic acid. The amount of the correction was ascertained as follows:—20 c.c. of diluted medium, containing 1·2 per cent. of lactic acid only, were distilled exactly as described above for acetic acid. A second distillation of 20 c.c. of the medium, containing 1·2 per cent. lactic acid and 0·5 per cent. acetic acid was carried out under exactly the same conditions. The number of cubic centimetres of 0·1N baryta neutralised by the lactic acid distilled over in the first experiment was then deducted from the number of cubic centimetres of baryta neutralised by the lactic and acetic acids distilled over in the second experiment. The difference gave the number of cubic centimetres of baryta neutralised by the acetic acid only. It was found that the amount of lactic acid distilled over under the standard conditions was fairly constant (corresponding to

1·9–2·1 c.c. of baryta), and a constant deduction of 2 c.c. of baryta was therefore made.

The residue left in the Claissen flask after the acetic acid had been distilled off was quantitatively removed and the lactic acid estimated as in Experiment 4. The result was, of course, too low, owing to the loss of the lactic acid which had been distilled over with the acetic acid (*i.e.*, 0·18 grm. per 100 c.c. of diluted medium). A small addition had therefore to be made to the results obtained by extraction.

Utilisation of Lactic and Acetic Acids present together.

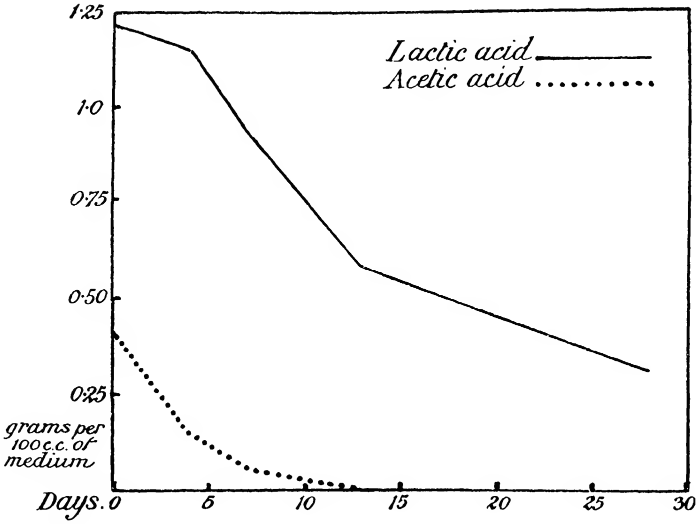
Day of experiment.	Acetic acid present; grm. per 100 c.c. of medium.	Acetic acid utilised; grm. per 100 c.c. of medium.	Lactic acid present; grm. per 100 c.c. of medium.	Lactic acid utilised; grm. per 100 c.c. of medium.
0	0·41	0·00	1·22	0·00
4	0·15	0·26	1·15	0·07
7	0·055	0·355	0·94	0·28
13	0·00	0·41	0·58	0·64
28	—	—	0·32	0·90

Two similar experiments were carried out with 1 per cent. glucose and 1·5 per cent. and 0·7 per cent. respectively of acetic acid (added as potassium acetate).

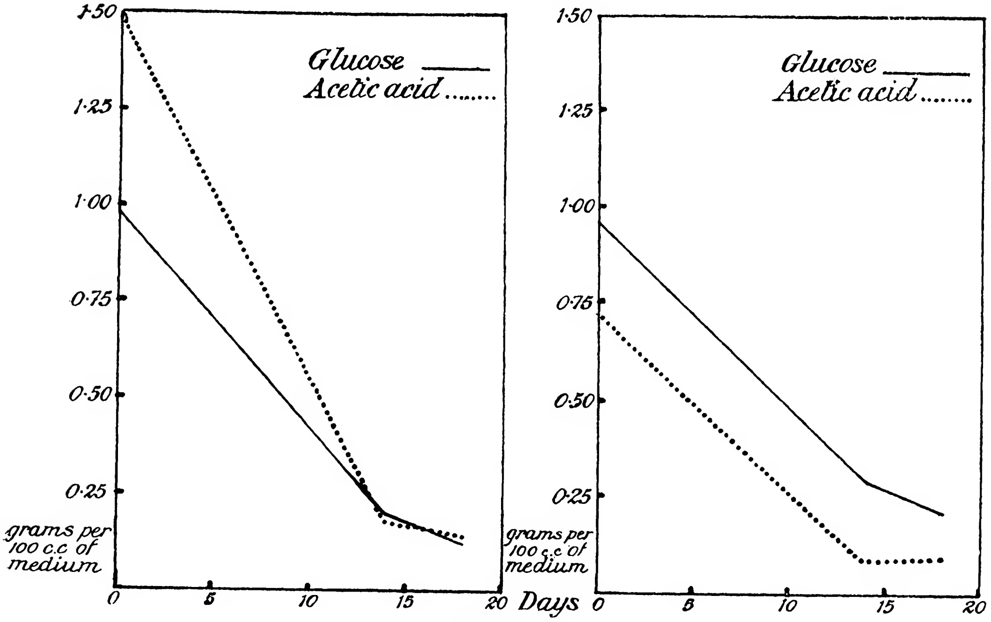
Utilisation of 1 per cent. Glucose and 1·5 per cent. Acetic Acid present together.

Day of experiment.	Acetic acid present; grm. per 100 c.c. of medium.	Acetic acid utilised; grm. per 100 c.c. of medium.	Glucose present; grm. per 100 c.c. of medium.	Glucose utilised; grm. per 100 c.c. of medium.
0	1·49	0·00	0·98	0·00
14	0·18	1·31	0·21	0·77
18	0·14	1·35	0·12	0·86
Utilisation of 1 per cent. glucose and 0·7 per cent. acetic acid present together.				
0	0·72	0·00	0·95	0·00
14	0·08	0·64	0·29	0·66
18	0·09	0·63	0·22	0·73

The above tables and accompanying graphs (Curves 4 and 5) show that, in the presence of lactic acid, or of glucose, the organism is capable of utilising the lactic acid completely.



Curve 4.



Curve 5.

Influence of Diet on the Chemical Composition of the Organism.

Having obtained growth on media with various organic constituents, it is of interest to notice what influence these substances have on the proportion of lipoids to nitrogenous material in the organism. The graph illustrating Experiment 1 shows that the lipoids and synthesised nitrogen rise to a maximum at a point closely corresponding in time to the disappearance of the organic constituents of the medium, and that after this point the lipoids decrease rapidly and the nitrogenous material very slowly. The point of maximum lipoid formation is taken as the stage at which the composition of the organism shall be estimated for purposes of comparison. The results are summarised in the following Table and shown graphically in fig. 2:—

Reference to fig. 2.	Organic constituents of medium.	Period of maximum lipoid formation in days.	Grm. in 100 c.c. of medium.		Ratio : Total lipoid / Nitrogen.
			Nitrogen synthesised by organism.	Total lipoids formed by organism.	
1	0·68 p.c. lactic acid...	10	0·019	0·016	0·84
2	1·4 p.c. lactic acid ..	11	0·026	0·020	0·78
3	1·2 p.c. ,, ,, .. 0·4 p.c. acetic acid ...	7	0·020	0·035	1·78
4	1 p.c. glucose	12	0·018	0·028	1·6
5	1 p.c. ,, ... 1 p.c. acetic acid	21	0·018	0·042	2·34
6	1 p.c. glucose	15	0·015	0·019	1·3
7	2 p.c. ,, ...	15	0·034	0·021	0·91

The results of the two experiments made with lactic acid alone in different amounts, approximate closely in the proportion of lipoid to nitrogen (fig. 2—1 and 2). When the organism is grown on a mixture of lactic and acetic acids, no more nitrogen is synthesised than on lactic acid alone, but the lipoid is increased by about 100 per cent. (fig. 2—3). A similar effect appears when

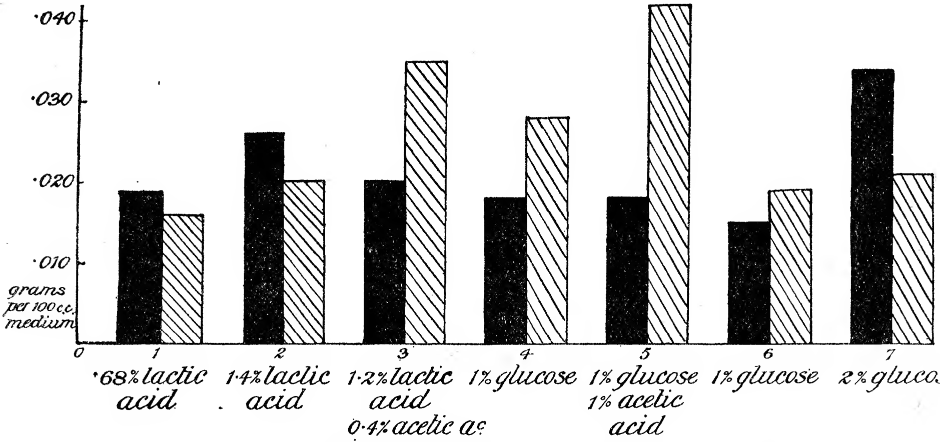


Fig. 2.—Diagram showing the influence of diet on the chemical composition of the Timothy grass bacillus.

Black blocks represent grams of nitrogen synthesised per 100 c.c. of medium. Shaded blocks represent grams of lipoid synthesised per 100 c.c. of medium.

acetic acid is added to a glucose medium (fig. 2—4 and 5); the added acetic acid does not affect the nitrogen synthesised (0·018 grm. in both cases), whilst the ratio of lipoids to nitrogen rises from 1·58 to 2·34.

It seemed possible that this result might not be due specifically to the acetic acid, but might be caused by the greater concentration of organic food stuff in the medium. Experiments made on 0·68 per cent. and 1·4 per cent. of lactic acid (fig. 2—1 and 2), and on 1·0 per cent. and 2·0 per cent. of glucose (fig. 2—6 and 7), show that this is not so. Increased concentration of food in each case results in an increased production both of synthesised protein and of lipoid material, *i.e.*, increased general growth. The production of protein is favoured rather more than that of fat, as is shown by the ratio of lipoids to nitrogen.

The inability to use acetic acid in the absence of carbohydrate or lactic acid forms at first sight an analogy to the inability of the higher animals to use fat in the absence of a minimum of carbohydrate. From this analogy, the authors were led to grow the bacillus on the sodium salts of propionic and butyric acids. Growth on both these proceeded readily in a manner comparable to growth on lactate and on glucose. The problem therefore appears to be one of some complexity. A detailed study of the growth of this organism on various simple straight-chain compounds is in progress, and will form the subject of a later communication.

SUMMARY.

The growth of the Timothy grass bacillus on a medium consisting of inorganic salts, including ammonia as the sole source of nitrogen, glucose, and sodium acetate, was followed in detail. The formation of protein nitrogen and fat (a phosphatide and a non-phosphatide fraction) were followed stage by stage, and correlated with the disappearance of glucose and acetate from the medium. Attempts were made to isolate intermediate decomposition products of glucose, but none were found.

The growth of the organism on possible intermediate products of the breakdown of glucose was then studied. The growth on lactic acid (as lactate) was very similar to that on glucose alone. The lactic acid could be completely utilised and the formation of protein and fat resembled that on a glucose medium. Growth on acetic acid (present as sodium acetate) was negligible in amount, and the acetic acid was not attacked by the organism. A modified method of estimating acetic acid is described. Growth on acetic and lactic acid (acetate and lactate) showed that the presence of lactic acid enabled the organism to utilise the acetic acid. Glucose also enabled the organism to utilise the acetic acid.

A comparison of the composition of the growth on these media showed that the acetic acid utilised in the presence of lactic acid or glucose served, not to increase the general growth of the organism, but to increase the proportion of lipoid material formed. This was shown to be a specific effect of the acetic acid, and was not merely due to greater concentration of carbonaceous food material.

Experiments were made to ascertain whether the behaviour of the bacillus on other straight-chain fatty acids resembled that on lactic acid (and glucose) or that on acetic acid. Growth on propionic acid and on butyric acid was like that on lactic acid, *i.e.*, the organism was able to grow on these compounds without the addition of other carbon compounds, and to synthesise both nitrogenous and fatty material.

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REFERENCES.

(1) Goris (Part I), Goris and Liot (Parts II and III), "Composition Chimique du Bacille Tuberculeux," 'Ann. de l'Institut Pasteur,' vol. 34, p. 497 (1920).

(2) Hopkins and Fletcher, "Lactic Acid in Amphibian Muscle," 'J. of Physiology,' vol. 35, p. 247 (1907).

(3) Van Slyke, "The Analysis of Proteins, etc.," 'J. Biol. Chem.,' vol. 10, p. 15 (1911).

(4) Wood and Berry, "A Rapid Method of Estimating Sugar," 'Proc. of the Cambridge Philosophical Society,' vol. 12, p. 97 (1903).